Multi-residue analysis of pharmaceuticals in Belgian surface water: a novel screening-to-quantification approach using large-volume injection liquid chromatography coupled to high-resolution mass spectrometry

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Abstract

The ever growing number of emerging micropollutants such as pharmaceuticals requests rapid and sensitive full-spectrum analytical techniques. Time-of-flight high-resolution mass spectrometry (TOF-HRMS) is a promising alternative for the state-of-the-art MS/MS instruments because of its ability to simultaneously screen towards a virtually unlimited list of suspect compounds and to perform target quantification. The challenge for such suspect screening is to develop a strategy which minimizes the false negative rate without restraining numerous false positives. At the same time, omitting laborious sample enrichment through large-volume injection ultra-performance liquid chromatography (LVI-UPLC) is advantageous avoiding selective preconcentration.

A novel suspect screening strategy was developed using LVI-UPLC-TOF-MS aiming the detection of 69 multi-class pharmaceuticals in surface water without the a priori availability of analytical standards. As a novel approach, the screening takes into account the signal intensity-dependent accurate mass error, hereby assuring the detection of 95% of pharmaceuticals present in surface water.

Subsequently, the validation and applicability of the full-spectrum method for target quantification of the 69 pharmaceuticals in surface water is discussed. Analysis of five Belgian river water samples revealed the occurrence of 17 pharmaceuticals in a concentration range of 17 ng L^{-1} up to 3.1 µg L^{-1}.

Introduction

Pharmaceuticals are emerging environmental micropollutants that receives increasing attention worldwide. Their continuous introduction into the environment, bio-recalcitrance, and intrinsic ability to interfere with organisms concern the scientific community for their potential ecotoxic effects on the environment, toxicity towards humans, and the selection of antibiotic resistance in bacteria (Kümmerer et al., 2009; 2010). No current legislative framework exists in the European context defining allowable concentrations for these potentially harmful pharmaceuticals in the environment. However, recently, the European Commission published a proposal concerning the review of the list of priority substances for the Water Framework Directive 2000/60/EC (Commission Decision, 2000) including a maximum annual average concentration of 100 ng L^{-1} for diclofenac in surface waters.

The growing interest towards screening and quantification of this diverse group of pharmaceuticals in all kinds of environmental samples requests advanced multi-residue analytical techniques. Full-spectrum high-resolution mass spectrometers (HRMS) such as magnetic sector, time-of-flight (TOF) and orbitrap instruments are therefore a promising alternative for the current state-of-the-art triple quadrupole (QqQ) MS/MS instruments. In MS/MS, typically a target analysis is performed on a predefined limited set of compounds of interest hereby depending on the availability of standards. In contrast, the full-spectrum HRMS approach has shown the potential to analyze and identify based on accurate mass a virtually unlimited number of analytes simultaneously and offers the ability for both suspect screening and target quantification (Chitescu et al., 2012; Díaz et al., 2011; Ibáñez et al., 2009; Krauss et al., 2010; K'oreje et al., 2012; Müller et al., 2011; Petrović et al. 2006). In suspect screening using full-spectrum HRMS instruments, there is no a priori need for analytical standards because the acquired chromatograms are searched for the exact ion masses of an unlimited list of suspect compounds within a certain mass tolerance. In a next stage, confirmation of the found suspects with analytical standards based on chromatographic retention time and/or fragment ions is possible and a target quantification can be performed through validation of only the limited set of confirmed compounds.

Achieving quantification of trace amounts is a challenge in environmental analysis. Usually, samples must be preconcentrated and a clean-up of interfering matrix compounds is necessary to enhance the method's performance limits using an enrichment step such as solid-phase extraction (SPE). These laborious sample preparations techniques can be omitted when applying large-volume injection (LVI, 250 µL) instead of the traditional 5 to 20 µL injections. As an advantage, unwanted selectivity induced by the preconcentration step can be avoided using the LVI approach.

Hence, we aimed to investigate and improve the potential of large-volume injection – ultra-performance liquid chromatography (LVI-UPLC) in combination with quadrupole time-of-flight (QTOF) HRMS for both rapid screening and target quantification of traces of pharmaceuticals. An optimized and validated novel and rapid analytical method for a broad variety of multi-class pharmaceuticals in surface water is presented, hereby aiming to screen and quantify traces down to a concentration of 100 ng L^{-1}.

To reach these goals, we investigated and optimized the determination of the accurate mass for qualitative analysis. Subsequently, the relationship between the mass error and the signal intensity was investigated allowing the establishment of a signal intensity-dependent mass error tolerance and hereby keeping the false negative screening rate at 5%. Finally, the results of a suspect screening and validated target quantification study on five Belgian river water samples are presented.

Methods

Analytical standards

Individual stock solutions of the 69 pharmaceuticals were prepared on a weight basis to a final concentration of 1 mg mL^{-1}. Daily, a standard mix of the pharmaceuticals was prepared in deionized water, and subsequently serial diluted to a final concentration of 5, 1, 0.5, 0.1, 0.05 and 0.01 µg L^{-1} in deionized (i.e. analytical standards) and river water.

Sampling and sample pretreatment

Five river water samples were collected in prerinsed amber glass bottles on five different locations along the Maas and the Albert channel, Belgium, and stored at 4°C in the dark for no longer than 24 hours prior to analysis. Prior to standard addition, river water samples were filtered through 1.5 µm glass microfiber filters (934-AH, Whatman) and subsequently 0.1% and 0.02% (v/v) formic acid was added to all samples for analysis in electrospray positive and negative ion mode, respectively.

Instrumental analysis

The analysis were performed using an ultra-performance liquid chromatography (UPLC) system Waters Acquity (Waters, Milford, MA, USA) equipped with an autosampler with 250 µL loop for large-volume injection and coupled to a Xevo G2 QTOF time-of-flight mass spectrometer with an orthogonal electrospray ionization (ESI) probe (Waters Corporation, Manchester, U.K.). Chromatographic separation was achieved with a UPLC Aquity HSS T3 150x2.1mm 1.8µm column (Waters, Milford, MA, USA).

Briefly, for analysis in electrospray positive ion mode, the mobile phase used was (A) water/acetonitrile 98:2 (v/v) with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. In electrospray negative ion mode, the mobile phase used was (A) water/acetonitrile 98:2 (v/v) with 0.01% formic acid and (B) acetonitrile. The elution gradient for both modes increased linearly from 3 to 98% B in 11 min. Subsequently, initial conditions were recovered. The total time for the chromatographic analysis was 19 min. The sample injection volume was 250 µL.

The QTOF mass spectrometer was operated at a resolving power of 20,000 at Full Width at Half Maximum (FWHM) acquiring profile data over an m/z range of 50-1200 Da. The data station operating software was Masslynx version 4.1 (Waters).

Results and discussion

Determination of the accurate mass for qualitative analysis

A first challenge in the analytical data processing is the accurate and precise determination of the mass of a detected compound. Raw high-resolution MS data are

profile data on which no post-processing is performed and where each scan in the chromatogram consists of a profile mass spectrum. The simplest methodology for the accurate mass determination reads the accurate mass directly from the raw profile data and takes the mass at the maximum intensity as the accurate mass. On the other hand, on post-processing the profile data by a centroiding algorithm, sticks replace the peaks of the profile mass spectrum and the mass attributed to each stick is the centroid. The result is thus a spectrum with sticks where the resolution of the spectrum peaks is eliminated. The question is how good such centroid algorithms are for finding the center of a mass spectrum peak in terms of mass accuracy and precision.

To compare and evaluate the performance of both methodologies, the correctness of the accurate mass extracted from the profile data is compared to that obtained after centroiding the spectra using the Automated Peak Detection (APD) algorithm in the Masslynx software. Therefore, spectra extracted from an assay with deionized water spiked with a set of 17 compounds at a concentration of 5 µg L^{-1} are used (mass range between 152 and 916 Da). For each compound, 5 scans were selected between the chromatographic peak apex and the peak tail in order to cover a wide range of signal intensities. The variability on the mass accuracy clearly improved for the APD centroiding algorithm compared to the profile data approach. A significant decline of the standard deviation by a factor of 2.3 (two-sided F-test, $p < 0.05$) was observed. Therefore, for qualitative analysis a better estimate of the accurate mass of a spectrum peak can be obtained after centroiding the profile data.

Development of an improved suspect screening strategy

For screening, extracted ion chromatograms are constructed utilizing an optimized mass window width of 50 ppm (Vergeynst et al., 2013) and the APD algorithm was used to determine the accurate mass of found peaks.

When screening a sample towards a list of suspect compounds, the error on the accurate mass must fall within certain boundaries (mass error tolerance) in order to restrain the detected compound as being the suspect one. In order to determine how wide this mass error tolerance should be when accepting a false-negative screening rate of 5%, a river water sample spiked with analytical standards of a sub-selection of 44 pharmaceuticals (data not shown) to 0.01, 0.05, 0.1, 0.5, 1 and 5 µg L^{-1} was analysed, resulting in a training dataset of 208 observations.

The variability of the accurate mass error showed to strongly decrease with increasing signal intensity (Figure 1). The multiplication of the mass error (ME) and the log-transformed signal intensities (i) of the training data is independent of the signal intensity and uniformly distributed. Hence, the variability of the mass error was modelled as: $ME \cdot \log(i) \sim N(0, \sigma^2)$.

This model permitted to draw the 95% confidence limits of the mass error as a function of the signal intensity (Figure 1). By screening an unknown sample, a smaller mass error tolerance will be applied for the positive conclusion of peaks with higher signal intensities or, in other words, the observations should fall within the 95% confidence limits in Figure 1 to be restrained.

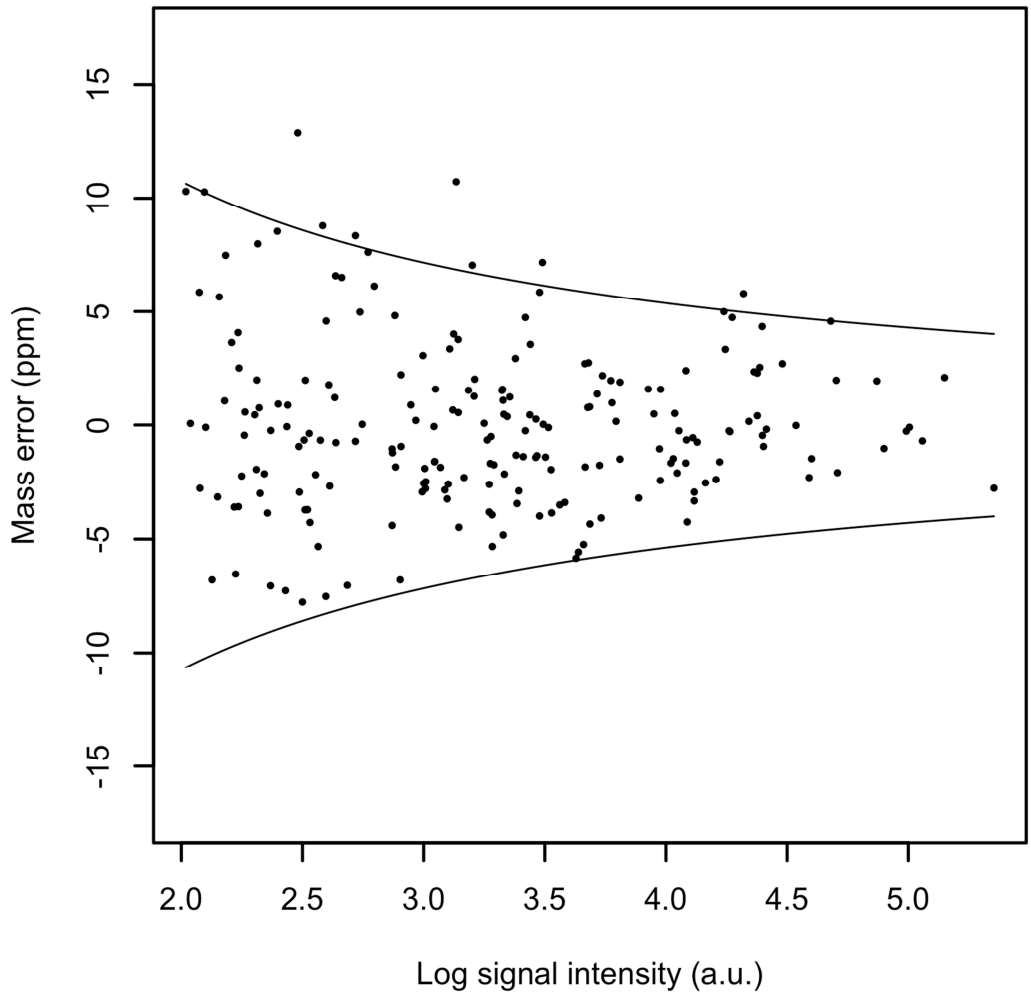

Figure 1. The variability of the mass error decreased inversely with the log-transformed signal intensity. The training dataset was used for the calculation of the 95% confidence limits.

Suspect screening and target quantification of multi-class pharmaceutical residues in Belgian river water

The applicability and performance of the developed optimized signal-intensity based suspect screening strategy was investigated by the analysis of five river water samples. By subsequent analysis of analytical standards of the suspect compounds, restrained peaks were confirmed when their retention time deviates not more than 1.96 x standard deviation, i.e. within the 95% confidence interval, from the retention time of the respective analytical standard.

In order to estimate both false negative and false positive screening rates, all found peaks in the surface water samples within a wider mass error tolerance of ±25 ppm are considered in a first step. The peaks restrained by the suspect screening (157 hits related to 37 different suspect compounds) fall within the 95% confidence limits. The signal intensity based screening showed a good performance with a false negative rate (i.e. peaks not-restrained by the suspect screening but confirmed by retention time) of 4.6%. Seven out of the 37 restrained suspect compounds were not confirmed by

retention time, resulting in a false positive rate of 10.1% (i.e. fraction of the 69 suspect compounds restrained but not confirmed by retention time).

The screening strategy thus revealed that in the 5 river water samples, 30 out of the 69 suspect pharmaceutical compounds were restrained and confirmed in at least one sample. Subsequent target quantification and full method validation revealed a concentration range from 17 ng L^{-1} to 3.3 µg L^{-1} in the analysed river water (data not shown). For 5 compounds (atenolol, caffeine, ibuprofen, roxithromycin and sotalol) the concentration range exceeded the level of 100 ng L^{-1} at least once. Wille et al. (2010) detected and quantified 8 pharmaceuticals in seawater (1 - 855 ng L^{-1}) and marine organisms from the Belgian coastal zone. To our knowledge, the results obtained in this study are the first validated ones for pharmaceuticals in Belgian fresh river waters.

Advantages of the novel screening-to-quantification approach

An important advantage of the developed suspect screening strategy is that there is no a priori need for analytical standards. For confirmation of the suspect screening results, only analytical standards of the restrained compounds are necessary and if the aim is also quantification, the validation of only the restrained and confirmed compounds is sufficient for a reliable quantification. Considering the 5 surface water samples focused in this study, mass traces related to 37 different suspect compounds were restrained in the chromatograms. Analyzing only these 37 compounds as analytical standards allows the confirmation based on the retention time, which was the case for 30 out of the 37 compounds. As a consequence, less analytical standards (37 instead of 69) are necessary and the workload for the full method validation can be reduced (30 instead of 69).

Large-volume injection showed to be a second important advantage of the presented rapid analytical screening and quantification technique. The chromatography takes 19 min and the analytical method requires no sample pretreatment (except for filtering the sample). This is in contrast with most published analytical methods for the analysis of micropollutants in surface water applying laborious and time-consuming SPE enrichment steps. Besides, sample enrichment techniques such as SPE preconcentrate compounds selectively and, as highlighted by Chitescu et al. (2012), achieving acceptable recoveries for all compounds is unlikely in multi-residue applications.

Further research and optimization of LVI in combination with the newest-generation and more sensitive full-spectrum HRMS is encouraged for suspect screening and quantification of micropollutants in surface water aiming at least a performance limit of 100 ng L^{-1}.

Conclusion

An innovative analytical method for screening and quantification of a set of 69 pharmaceutical compounds in river water based on LVI-UPLC coupled to a quadrupole time-of-flight (QTOF) MS was developed and validated.

A novel suspect screening strategy was established, assuring a false negative rate of 5% by modelling the variability of the signal intensity-dependent accurate mass error. A first screening of five Belgian river water samples revealed the occurrence of 30 out of 69 suspect pharmaceuticals (antibiotics, analgesics, antidepressants, alkylating

agents, anti-inflammatories, etc.). The validated target quantification confirmed the presence of the pharmaceuticals in a concentration range of 17 ng/L up to 3.3 µg/L.

The novel screening-to-quantification approach allows screening without the a priori availability of analytical standards. The applicability of this approach has been established as 30 out of the 37 compounds that were restrained by the screening strategy could be confirmed. As a consequence, less analytical standards (37 instead of 69) are necessary and the workload for the validation can be reduced (30 instead of 69).

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